EXHIBIT C3

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The production of soluble pyrroloquinoline quinone glucose dehydrogenase by *Klebsiella pneumoniae*, the alternative host of PQQ enzymes

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Abstract

Klebsiella pneumoniae, which produces PQQ and is available for use with a conventional expression vector system, was selected as the host strain for soluble PQQ glucose dehydrogenase (PQQGDH-B) production. The recombinant *K. pneumoniae* expressed PQQGDH-B in its holo-form at about 18 000 U l^{-1}, equal to that achieved in recombinant *Escherichia coli*. The signal sequence of recombinant PQQGDH-B produced by *K. pneumoniae* was correctly processed. *K. pneumoniae* can become an alternative host microorganism not only for PQQGDH-B production but also for recombinant PQQ enzymes production.

Introduction

Glucose dehydrogenases (GDHs) possessing pyrroloquinoline quinone (PQQ) as the prosthetic group are widely distributed within Gram-negative bacteria, mainly as the membrane-bound enzyme (PQQGDH-A). A similar enzyme, the soluble PQQ glucose dehydrogenase (PQQGDH-B) is structurally quite different from PQQGDH-A and so far has only been found in the periplasm of the bacterium *Acinetobacter calcoaceticus* (Matsushita *et al.* 1995).

PQQGDH-B is a homodimeric enzyme containing PQQ and Ca^{2+}. It is recognized as an ideal enzyme for a mediator-type glucose sensor since it has a high turnover number and the electrochemical cofactor regeneration is not affected by the presence of O_2 in the samples (Kost *et al.* 1998, Schmidt 1997, Ye *et al.* 1993). The expression of recombinant PQQGDH was first reported in a PQQ-producing hosts, such as *Acinetobacter* strains and *Pseudomonas* strains, utilizing broad host range vectors (Cleton-Jansen *et al.* 1988, 1990). We previously reported the production of recombinant *Escherichia coli* PQQGDH and *A. calcoaceticus* PQQGDH-A using *E. coli* (Sode *et al.* 1994, 1996). Since *E. coli* cannot synthesize PQQ, PQQGDH is produced in the apo-form. In order to produce holo-GDH in recombinant *E. coli*, cultivation should be carried out in the presence of PQQ and bivalent metal in the medium. However, the high cost of PQQ may prevent its use as a medium component for the large-scale preparation of recombinant PQQGDHs. We previously showed that the production of recombinant PQQGDH-A occured in the holo-form, using an *E. coli* strain harboring the heterologous *pqq* operon from *K. pneumoniae* (Sode *et al.* 1996). However, this system had the problem that the population of *E. coli* cells harboring both the GDH structural gene and *pqq* operon decreases during cultivation. Therefore, a host microorganism which is able to produce PQQ and is available for use with a conventional expression vector system is ideal for the production of recombinant PQQGDH and also for other PQQ enzymes.

Klebsiella pneumoniae is an enterobacterium and can synthesize PQQ (Meulenberg *et al.* 1990). In addi-

tion, there are several studies which demonstrate that *E. coli* vector systems are functional in *K. pneumoniae*, such as *oriC* for replicants and *lac* promoters for gene expression (Harding *et al.* 1982, Kleiner *et al.* 1988). These results suggest that an *E. coli* expression vector system can be utilized also in *K. pneumoniae*. Therefore, it is expected that by using *K. pneumoniae* as the host strain, efficient holo PQQGDH-B production is possible.

In this study, we report the production of recombinant PQQGDH-B, using *K. pneumoniae*.

Material and methods

Bacterial strain, plasmids and culture media

Klebsiella pneumoniae NCTC418 (Robinson & Tempest 1973) and *Escherichia coli* PP2418 (Cleton-Jansen *et al.* 1990) were used throughout these experiments. For the production of PQQGDH-B, plasmids, pGB and pGBK were used. pGB was constructed by inserting the structural gene of PQQGDH-B of *A. calcoaceticus* LMD79.41, under the control of the *trc* promoter, present in the expression vector pTrc99A (Amersham Pharmacia, Sweden). pGBK was derived from pGB by inserting a kanamycin resistance gene block of pUC4K (Amersham Pharmacia, Sweden) in the ampicillin resistance gene (Figure 1). The transformation of *K. pneumoniae* was carried out by electroporation using Gene Pulser (BioRad, California, USA). The field strength was 12.5 kV cm^{-1}.

E. coli PP2418/pGB was cultured in a Luria broth with 25 mg ml^{-1} ampicillin, 25 mg ml^{-1} chloramphenicol, 1 mM $CaCl_2$ and 600 nM PQQ. *K. pneumoniae* NCTC418/pGBK was cultured in a Luria broth with 25 mg ml^{-1} kanamycin and 1 mM $CaCl_2$.

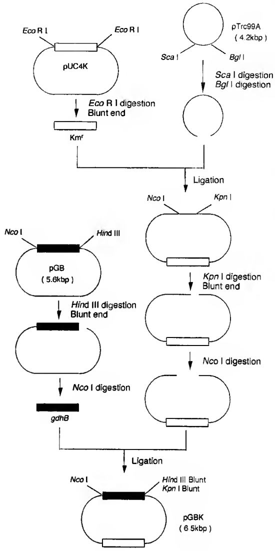

Fig. 1. Construction of an expression vector, pGBK.

PQQGDH-B expression in K. pneumoniae

The cultures were prepared in 3 ml of media containing 1 mM $CaCl_2$ and then incubated on a reciprocating shaker overnight at 37 °C and 80 rpm. After 4 h, 0.3 mM IPTG was added to induce the expression of PQQGDH-B. The cells were harvested after 12 h and GDH activity and protein concentration were measured.

Seed cultures (150 ml × 2) were prepared in two 500-ml conical-shaped flasks and incubated in a reciprocating shaker overnight at 37 °C and 120 rpm. A batch culture was prepared at 37 °C in a 10-l jar fermenter (Mitsuwa Bio Systems, Japan) containing 7 l of medium by inoculating the above-mentioned seed culture. Aeration and agitation were controlled at 1 vvm and 800 rpm, respectively. The pH was not controlled. After 2 h, 0.3 mM of IPTG was added to induce the expression of PQQGDH-B.

The amount of produced PQQ by *K. pneumoniae* was measured as previously reported (Sode *et al.* 1996).

Table 1. Glucose dehydrogenase activity of *E. coli* and *K. pneumoniae*.

		Activity (U mg^{-1} protein)			
		PP2418 (*E. coli*)	PP2418/pGB (*E. coli*)	NCTC418	NCTC418/pGBK
+ PQQ	Glucose (100 mM)	0	4.6	0.2	6.3
	Lactose (25 mM)	0	3.1	0	4.2
− PQQ	Glucose (100 mM)		0		4.0
	Lactose (25 mM)		0		2.5

+ PQQ: 1 mM $CaCl_2$ and 5 mM PQQ was added when GDH activity was measured.
− PQQ: 1 mM $CaCl_2$ was added when GDH activity was measured.

Analytical methods

Growth was monitored by measuring the optical density at 660 nm (1 OD_{660} unit = 0.4 mg dry cell wt ml^{-1}). The amount of PQQGDH production in recombinant *E. coli* and recombinant *K. pneumoniae* was measured as follows. One ml of culture was centrifuged for 5 min at 5000 × *g*. The centrifuged cells were washed with 10 mM MOPS buffer (pH 7) three times and resuspended in 500 μl 10 mM MOPS buffer (pH 7). This sample, 30 μl, was mixed with 10 μl 10% SDS and boiled. Then, the protein concentration was measured using a DC Protein Assay Kit (BioRad, CA, USA). Ten μl of the resuspended cells were used for the GDH assay. This sample was mixed with 80 μl of 10 mM MOPS buffer (pH 7), 5 mM PQQ, (or absence of PQQ), 1 mM $CaCl_2$, 60 μM DCIP and 1 mM PMS and the rate of decrease in absorbance at 600 nm was measured.

Purification, SDS-PAGE and N*-terminal amino acid sequence analysis*

Purified enzyme was prepared as follows. The cells were harvested after the late log phase, resuspended in 10 mM potassium phosphate buffer (pH 7.0) and disrupted in a French Press (110 MPa). The sample was then subjected to ultracentrifugation (160500 × *g*, 1.5 h, 4 °C), following the dialysis in 10 mM potassium phosphate buffer (pH 7.0). The obtained supernatant was applied to CM-Toyopearl 650 M cation exchange column (Tosoh, Japan) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 0–0.2 M NaCl in 10 mM potassium phosphate buffer (pH 7.0).

The molecular mass of the sample was measured by SDS-PAGE using Phast System (Amersham Pharmacia, Sweden). The prepared PQQGDH-B sample was subjected to SDS-PAGE separation and the band corresponding to PQQGDH-B was blotted to the PVDF membrane. This sample was used for the *N*-terminal amino acid sequence analysis by PPSQ-10 (Shimadzu, Japan).

Results and disciussion

We first confirmed the expression of PQQGDH-B in *K. pneumoniae* NCTC418/pGBK. The results are given in Table 1. *E. coli* PP2418, the *gdh*$^-$ mutant, showed no GDH activity. *E. coli* PP2418/pGB expressed PQQGDH-B and showed GDH activity with glucose and lactose. *K. pneumoniae* NCTC418/pGBK showed high glucose and lactose oxidation activity. *K. pneumoniae* NCTC418 has endogenically membrane-bound PQQGDH(PQQGDH-A) (Neijssel *et al.* 1983). But the dehydrogenase activity of *K. pneumoniae* NCTC418 measured against glucose was 0.20 U mg^{-1}, over 30-fold less than the activity of *K. pneumoniae* NCTC418/pGBK. Furthermore, *K. pneumoniae* GDH does not utilize lactose as the substrate. Therefore, the observed dehydrogenase activity of *K. pneumoniae* NCTC418/pGBK for glucose and lactose suggest that PQQGDH-B was expressed in its active form in *K. pneumoniae* NCTC418/pGBK.

When GDH activity was measured without the addition of PQQ, *E. coli* PP2418/pGB showed no GDH activity because *E. coli* did not produce PQQ. On the other hand, *K. pneumoniae* NCTC418/pGBK showed GDH activity, when the GDH activity measurement was carried out without PQQ being added. Therefore, PQQGDH-B was produced in holo-form in *K. pneumoniae* NCTC418/pGBK. However, GDH activity without the addition of PQQ at the time of the GDH activity measurement was about 60% of the GDH activity with PQQ added. This suggests that all

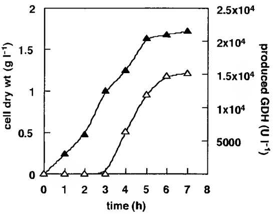

Fig. 2. Time course for the production of PQQGDH-B of *E. coli* PP2418/pGB. Cell dry wt (▲); enzyme activity (△).

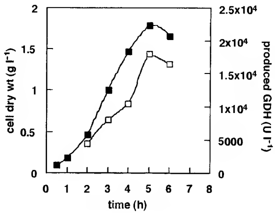

Fig. 3. Time course for the production of PQQGDH-B of *K. pneumoniae* NCTC418/pGBK. Cell dry wt (■); enzyme activity (□).

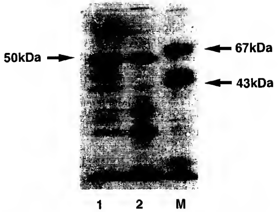

Fig. 4. SDS-PAGE of PQQGDH-B produced in recombinant *E. coli* and *K. pneumoniae*. 1: *E. coli* PP2418/pGB; 2: *K. pneumoniae* NCTC418/pGBK; M: Molecular weight markers.

PQQGDH-B did not appear in holo-form. The amount of PQQ produced would be insufficient to make all PQQGDH-B in holo-form.

We next compared the PQQGDH-B specific activity in recombinant *K. pneumoniae* and *E. coli*. *K. pneumoniae* NCTC418/pGBK was cultivated in a 10-l jar fermenter containing 7 l of Luria broth. The cultivation was performed in the presence of 1 mM $CaCl_2$. *E. coli* PP2418/pGB was cultivated under the same condition as *K. pneumoniae* NCTC418/pGBK except for the fact that the culture contained 600 nM PQQ. The results are given in Figures 2 and 3. In *E. coli* PP2418/pGB, the amount of PQQGDH-B produced was about 15000 U l^{-1}. In *K. pneumoniae* NCTC418/pGBK, the activity of PQQGDH-B produced was about 18000 U l^{-1}, similar to that produced in *E. coli*. The plasmids used for PQQGDH-B expression are different between *K. pneumoniae* and *E. coli* because antibiotic resistance is different. The *K. pneumoniae* strain used in this study carried a gene for ampicillin resistance, therefore the expression with pGB could not be utilized. Considering that this *K. pneumoniae* strain is kanamycin sensitive, we introduced a Km^r cassette from pUC4K, and inserted it into the Amp^r gene of pGB, to be construct pGBK.

In the cultivation, the difference of the kinetics of PQQGDH-B production between *E. coli* and *K. pneumoniae* was observed. In *E. coli* PP2418/pGB, expression of PQQGDH-B was repressed for first 3 h and was induced after IPTG addition (Figure 2). In contrary, in *K. pneumoniae* NCTC418/pGBK, PQQGDH-B was expressed before IPTG addition (Figure 3). It seems that the repression by $lacI^q$ did not function in *K. pneumoniae*. However, Kleiner *et al.* (1988) reported that *tac* promoter and $lacI^q$ are functional in *K. pneumoniae*. Therefore, the difference of the kinetics of PQQGDH-B production between *E. coli* and *K. pneumoniae* observed in this study is likely because that the vector we used in this study might harbor some mutations on $lacI^q$ and/or *tac* promoter.

Since the inserted DNA in pGBK contains not only the structural gene for PQQGDH-B but also the leader sequence for this periplasmic protein, we investigated whether correct processing had occurred. The purified PQQGDH-B from recombinant *K. pneumoniae* gave a band of about 50 kDa on SDS-PAGE, which was the same as PQQGDH-B produced in recombinant *E. coli* (Figure 4). The sequencing of *N*-terminal amino

acids for the purified PQQGDH-B from recombinant *K. pneumoniae* revealed that it had a sequence identical to that deduced from the structural gene. The Km value of PQQGDH-B from recombinant *K. pneumoniae* for glucose was 20 mM. This value was almost same as the Km value of PQQGDH-B from recombinant *E. coli* for glucose as substrate (25 mM). These facts support the conclusions that correct processing of PQQGDH-B had occurred in recombinant *K. pneumoniae*.

We conclude that the use of recombinant *K. pneumoniae* allowed the production of a high level of PQQGDH in holo-form. In laboratory scale, the cost of PQQ accounted for about 30% of the total cost for the medium. The PQQGDH-B production using *K. pneumoniae* was advantageous in cost, although the amount of PQQ produced was not enough to make all of the PQQGDH-B in holo-form. *K. pneumoniae* can be an alternative host microorganism not only for PQQGDH-B production but also for recombinant PQQ enzymes production.

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